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CAFFEINE DEGRADATION BY YEASTS ISOLATED FROM CAFFEINE CONTAMINATED SAMPLES

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ABSTRACT
Five yeast species isolated from caffeine contaminated samples were screened for their capability of caffeine degradation. Based on the degradation efficiency, isolate was selected and identified as *Trichosporon asahii* which showed 60 % degradation of caffeine in 72 hours when caffeine was utilized as the sole carbon and nitrogen source. The influence of various factors such as pH, temperature, shaking speed, inoculum size, carbon source, nitrogen source and initial caffeine concentration on caffeine degradation were studied. The optimum growth conditions for caffeine degradation by *T. asahii* were found to be pH 6.5, temperature 28 °C, shaking speed 120 rpm, inoculum size 4 % (w/v) with initial caffeine concentration 2 gm/l and 100 % degradation was achieved within 96 hours in the presence of sucrose (5 gm/l). The addition of external nitrogen sources viz. sodium nitrite and sodium nitrate decreased the caffeine degradation to 25 % and 30 % respectively. This is the first report on caffeine degradation using yeast cells.

KEYWORDS: Assimilation; Carbon sources; Decaffeination; Nitrogen sources; *Trichosporon asahii.*

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid naturally occurring in coffee and cocoa beans, cola nuts, and tea leaves. Excessive consumption of caffeine through beverages results in a number of health problems like adrenal stimulation, irregular muscular activity, cardiac arrhythmias, osteoporosis and increased heart output (Schuh et.al., 1997). Excess caffeine is reported to cause mutation, inhibition of DNA repairs and inhibition of adenosine monophosphodiesterase (Blecher et.al., 1977) and during pregnancy causes malformation of foetus (Srisuphan et.al., 1986). Due to the known adverse effects of caffeine in the widely consumed beverages like coffee and tea, a caffeine free product is desirable. Apart from health effects, caffeine degradation is important from environment point of view. Disposal of coffee processing industrial waste into lakes makes drinking water non-potable (Buerge et.al., 2003). The presence of caffeine in soil also affects soil fertility as it inhibits seed germination and growth of seedlings (Friedman et.al., 1983; Batish et.al., 2008). Though coffee pulp and husk are rich in carbohydrates and proteins, the presence of antinutritional factors such as caffeine, polyphenols, and tannins restricted its use as animal feed (Mazzafera et.al., 1994).

Conventional methods of decaffeination usually involve the use of decaffeinating agents such as methylene chloride, ethylacetate, charcoal, triglycerides and supercritical CO_2. These conventional methods are expensive, toxic and non-specific to caffeine. In this regard biodecaffeination using microbes has been considered more suitable than currently used chemical methods. Some bacteria and fungi, viz. *Pseudomonas* sp. (Woolfolk, 1975), *Serratia* sp. (Mazzafera et.al., 1994), *Stemphyllium* sp. (Kurtzman et.al., 1971), *Klebsiella* and *Rhodococcus* sp. (Madyastha et.al., 1998), *Aspergillus* and *Penicillium* sp. (Hakil et.al., 1998), have been reported to be capable of degrading caffeine. But reports are scanty regarding the potentiality of yeast as caffeine degrader.

In this study, we have isolated five yeast species from caffeine contaminated samples. A preliminary study on the effects of various growth parameters on caffeine degradation process was conducted using the selected isolate *Trichosporon asahii*. The ability of *T. asahii* to degrade caffeine was found to be greatly influenced by sugars, nitrogen sources, pH and inoculum size.

MATERIALS AND METHODS

Chemicals

Caffeine (>99% pure) was purchased from Merck Limited, Mumbai, India. All other chemicals are of analytical grade procured from Himedia Limited, Mumbai, India and SRL Chemicals Limited, Mumbai, India.

Media

Caffeine liquid medium containing (gm/l): K_2HPO_4, 0.8; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.1; $FeSO_4.7H_2O$, 0.005; yeast extract 0.2 and caffeine, 5.0 was used as the growth medium, for enrichment and screening. YEPD (Yeast Extract Peptone Dextrose) as well as caffeine liquid medium were used for induction of the organisms by varying caffeine concentration. Complex media such as Yeast Carbon Base (YCB) and Yeast Nitrogen Base (YNB) were used for assimilation experiments. Caffeine liquid medium containing sucrose (5gm/l) was used for the degradation studies. For solid medium, agar (20gm/l) was added to caffeine liquid medium. The initial pH of the medium was adjusted to 6.0.

Screening and identification of yeasts degrading caffeine

Soil from coffee garden, coffee pulp, husk, pulp compost and coffee industrial effluent were collected from Coffee Board, Yercaud (India). These samples were used for the isolation of yeasts that degrade caffeine, through an enrichment culture technique. The isolates were maintained and subcultured in the caffeine agar medium contained (gm/l): Dextrose 5.0; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; yeast extract, 0.5 and caffeine, 3.0. Yeasts were identified to the species level using VITEK 2 compact yeast card reader with the software version: 03.01 from Council for Food Research and Development (CFRD), Kerala, India.

Growth of cells and induction for caffeine degradation

A loop full of actively growing culture of the yeasts was transferred to 100 ml of YEPD broth containing 3 gm/l caffeine and incubated at 30 °C in an orbital shaker for 48 h. A 5 % (v/v) of the 48 h grown pre-inoculum was transferred to 100 ml of YEPD broth containing 3 gm/l caffeine and grown under the same conditions.

Samples were drawn at known intervals of time for the measurement of cell growth. Biomass accumulated after 48 h was harvested by centrifuging at 20,000 × g for 5 minutes at 0-4 °C to form a pellet. The biomass pellet was aseptically transferred into 250 ml flask containing 100 ml of caffeine liquid medium with 5 gm/l caffeine and incubated at 30 °C in an orbital shaker for 4 days for inducing cells to degrade caffeine. These induced cells were harvested by centrifugation as before. The cells were washed several times with phosphate buffer to remove residues of caffeine. Three grams of these induced cells were suspended in phosphate buffer, which were used for caffeine degradation experiments.

Assimilation experiments

Minimal media YNB and YCB were prepared according to the manufactor's instructions and supplemented with 5 and 1 gm/l caffeine for testing the utilization of this compound as carbon source and nitrogen source respectively by the yeast species. Control experiments were performed with 1 gm/l ammonium sulphate as sole nitrogen source, in YCB, or with 5 gm/l dextrose as sole carbon source, in YNB.

Caffeine degradation experiments

The induced cell biomass was aseptically transferred to caffeine liquid medium (100 ml) containing 5 gm/l caffeine and incubated at 30 °C in an orbital shaker at 120 rpm for 4 days. Aliquot samples each of 1.5 ml were drawn at different time intervals, centrifuged and the supernatants were analyzed for caffeine content. Studies on the effect of pH (4-8), temperature (20-40 °C) and shaking speed (80,100, 120, 140 and 160 rpm) were carried out by incubating the induced cells in caffeine liquid medium containing 5 gm/l caffeine, which was adjusted to different pH, temperature and shaking speed.

For studying the effect of inoculum on caffeine degradation, induced cell suspension was added to caffeine medium containing 5 gm/l caffeine so as to obtain inoculation levels of 1.0, 2.0, 3.0, 4.0, and 5.0 % (w/v) (based on wet weight). In order to study the effect of carbon sources different carbon sources viz., dextrose, fructose, sucrose , maltose and lactose were added to the caffeine medium at 5 gm/l concentration and the time course of cell growth and caffeine degradation were measured. In order to study the effect of nitrogen sources on caffeine degradation, caffeine medium was supplemented with different nitrogen sources *viz*., ammonium sulphate, sodium nitrate, ammonium chloride, sodium nitrite and urea at 1 gm/l concentration. Studies on the effect of caffeine concentration on degradation efficiency were carried out by incubating the induced cells in caffeine liquid medium containing caffeine in the range of 1-12 gm/l. Samples were drawn at known intervals of time and analyzed for caffeine content. All experiments were performed in triplicates under identical conditions and the data presented are mean of triplicates.

Analytical determinations

Cell growth in the medium was monitored by measuring the optical density at 600 nm (OD_{600} of 0.5 corresponds to 0.586 gm dry weight/liter). Caffeine was estimated by UV-visible spectrophotometer (Hitachi U-2800). Absorbance was measured at 253 nm. Percentage of caffeine degradation was calculated as follows:

$$\text{Caffeine degradation (\%)} = (a-b)\times\frac{100}{a} \qquad (1)$$

where *a* is the initial caffeine concentration and *b* is the residual caffeine concentration.

RESULTS AND DISCUSSION

Screening and identification of yeasts degrading caffeine

Five isolated yeast species capable of growing on caffeine agar medium were selected for decaffeination studies. The isolated yeast species were identified as *Trichosporon asahii, Candida tropicalis, Candida krusei, Candida inconspicua* and *Kloeckera japonica* using vitek2compact yeast card reader.

Among the five yeast isolates, *Trichosporon asahii* showed higher growth (Fig. 1) and efficiently utilized 60 % of 5.0 gm/l of caffeine in the medium in 96 hours compared to other isolates which were able to utilize less amount of caffeine in the same time as shown in Figure 2.

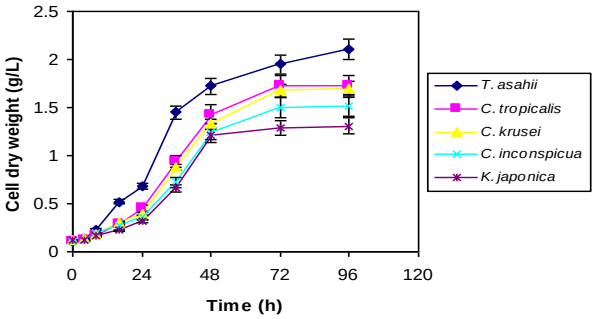

Fig. 1 Measurement of biomass of yeast species in caffeine liquid medium (with 5 gm/l of initial Caffeine concentration)

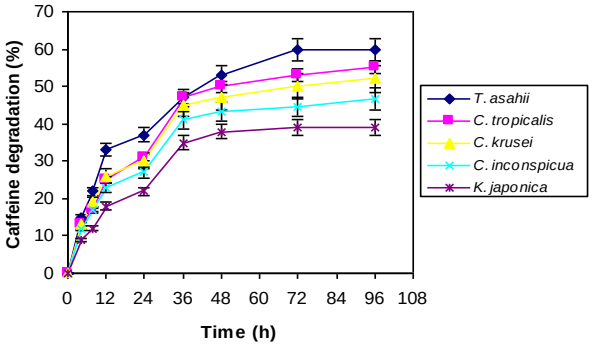

Fig. 2 Degradation of caffeine by yeast species incubated in caffeine liquid medium containing 5gm/l caffeine.

Assimilation experiments

Caffeine was tested as sole carbon or nitrogen source by determining the growth of the yeast cells in minimal media supplemented with 1 gm/l and 5 gm/l caffeine in YCB and YNB respectively. Appropriate controls, as described in Section 2, were simultaneously run. Figure 3 showed that caffeine behaved as sole source of nitrogen in YCB medium, as similar growth was observed with ammonium sulphate. In YNB containing caffeine as sole carbon source, relatively less growth was observed (Fig. 4). This result confirmed that caffeine can be assimilated as sole nitrogen source by all the yeast species but to a limited extent as sole carbon source.

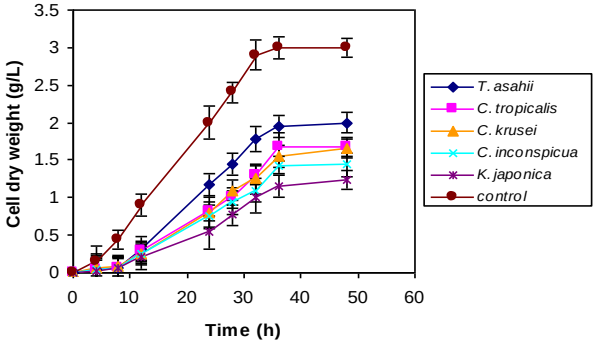

Fig. 3 Growth curves of yeast isolates obtained in YCB containing 1 gm/l caffeine as nitrogen source. Controls were done in the presence of 1 gm/l ammonium sulphate.

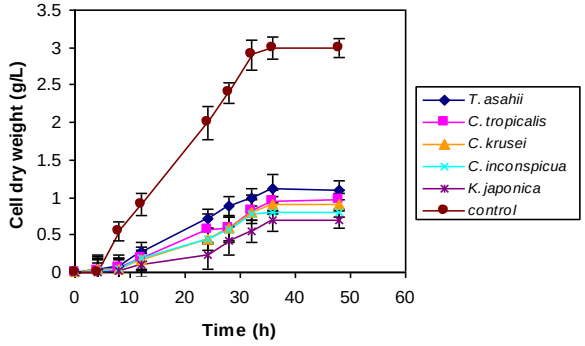

Fig. 4 Growth curves of yeast isolates obtained in YNB containing 5 gm/l caffeine as carbon source. Controls were done in the presence of 5 gm/l glucose.

Based on the above experimental results, *Trichosporon asahii* was found to be the most efficient utilizer of caffeine, so it was selected and used for further studies.

Effect of growth parameters on caffeine degradation by *Trichosporon asahii*

To study the effect of parameters viz. pH, temperature, shaking speed, inoculum size, carbon sources and nitrogen sources on caffeine degradation, yeast was grown in caffeine liquid medium containing 5 gm/l of caffeine in 250 ml conical flasks at 120 rpm and 30 °C. The same medium with varying concentrations of caffeine was used to study the effect of initial caffeine concentration on degradation. The experiments were performed in triplicates under identical conditions and the average result had a standard deviation varying between ±4% and ±8% caffeine degradation about the mean.

Experiments were performed to study the effect of different carbon sources on caffeine degradation process. Among all the tested carbon sources, sucrose was found to be the best (Table 1). Correspondingly, there was an increase in the biomass also. The maximum degradation percentage attained by *T. asahii* was 67 %. Similar result was noted in case of some strains of *Aspergillus* and *Penicillium* sp. where sucrose was supplemented as carbon source during caffeine degradation (Hakil et.al., 1998). In the case of bacterial systems, *Serratia marcescens* and *Pseudomonas putida* degraded caffeine in the absence of glucose or sucrose or any other carbohydrate in the medium (Woolfolk, 1975; Mazzafera et.al., 1994). However, it has been reported that certain *Pseudomonas* sp. and other bacteria degraded caffeine in the presence of additional carbon source (Madyastha et.al., 1998; Asano et.al., 1993). In *Pseudomonas* sp. GSC 1182, the presence of glucose inhibited caffeine degradation and the disaccharides sucrose and lactose enhanced caffeine degradation (Gokulakrishnan et.al., 2007).

The external nitrogen sources inhibited the caffeine degrading capability of the yeast cells. The maximum degradation percentage attained was very low (45 %) as shown in Table 1. However, it is to be noted that in the presence of additional nitrogen source, the biomass production was increased. This showed that when simple nitrogen source was present, caffeine was not used by the yeast cells. In contrast, if the simple nitrogen source was not enough to allow total carbon assimilation, caffeine could be degraded after the simple nitrogen get metabolized. Similar results were reported for fungi viz. *Penicillium* (Roussos et.al., 1994) and *Aspergillus* (Hakil et.al., 1999) species. Gokulakrishnan et al. (2007) reported that an external supply of the nitrogen sources (ammonium sulphate and urea) inhibited the degradation of caffeine in *Pseudomonas* sp. GSC 1182.

Table 1 Effect of various carbon and nitrogen sources on caffeine degradation by *T. asahii*

Time (h)	Amount of caffeine degraded (%)±standard deviation about the mean					Cell dry weight (gm/l) ±standard deviation about the mean				
	Effect of various carbon sources									
	D	*F*	*M*	*S*	*L*	*D*	*F*	*M*	*S*	*L*
0	0	0	0	0	0	0.11±0.01	0.12±0.01	0.11±0.01	0.11±0.01	0.13±0.01
24	25±4	19±5	21±6	24±5	18±5	0.50±0.03	0.40±0.03	0.35±0.02	0.99±0.02	0.38±0.02
48	37±5	25±7	32±4	52±6	23±7	0.99±0.04	1.19±0.04	1.18±0.03	1.43±0.05	0.90±0.03
72	44±6	43±4	40±5	59±4	28±8	1.40±0.02	1.55±0.04	1.49±0.04	1.66±0.04	1.42±0.04
96	56±5	51±6	49±6	64±5	39±6	2.09±0.01	2.00±0.01	2.12±0.03	2.20±0.03	2.00±0.03
120	60±7	55±5	55±5	67±4	50±4	2.29±0.03	2.24±0.01	2.22±0.05	2.35±0.02	2.19±0.03
	Effect of nitrogen sources									
	AS	*SN*	*AC*	*Sn*	*U*	*AS*	*SN*	*AC*	*Sn*	*U*
0	0	0	0	0	0	0.11±0.01	0.12±0.02	0.11±0.02	0.11±0.05	0.11±0.01
24	10±5	11±5	12±5	10±5	10±5	0.32±0.02	0.35±0.02	0.42±0.03	0.36±0.04	0.19±0.02
48	13±7	13±5	12±7	15±5	15±5	0.89±0.03	0.98±0.04	1.20±0.02	0.78±0.02	0.70±0.04
72	25±6	21±7	24±6	17±6	23±6	1.11±0.04	1.23±0.05	1.24±0.05	1.03±0.03	1.20±0.04
96	30±5	24±6	35±7	21±6	25±8	1.45±0.01	1.36±0.03	2.12±0.04	1.38±0.03	1.34±0.05
120	35±8	30±5	45±6	25±5	31±6	2.30±0.03	2.04±0.02	2.65±0.01	1.67±0.02	2.04±0.02

D- Dextrose; F- Fructose; M- Maltose; S- Sucrose; L- Lactose; AS- Ammonium sulphate; SN- Sodium nitrate; AC- Ammonium chloride; Sn- Sodium nitrite; U- Urea.

The effect of pH on caffeine (5 gm/l) degradation was studied by varying the initial pH between 4 and 8. *T. asahii* exhibited the maximum caffeine-degradation ability at pH 6.5. Maximum degradation of caffeine (69 %) was attained at 120 h resulting in higher biomass (Table 2). The degradation percentage was significantly less at pH 4.0 and 8.0. In further studies the initial pH of the medium was adjusted to 6.5.

The effect of temperature on caffeine degradation is shown in Table 2. The highest degradation was exhibited at a temperature 28 °C after which there was a decline. There was 73 % conversion of caffeine after 120 h of incubation. The biomass production was also maximum at 28 °C. The degradative ability of *T. asahii* was also fairly good at 20, 25 and 35 °C. At relatively higher temperature of 40 °C, the degradation of caffeine was less which was about 40 %.

The effect of agitation on cell growth and caffeine degradation by *T. asahii* was studied by varying the shaking speed at 80, 100, 120, 140 and 160 rpm. The results showed that there was no significant difference in the cell biomass production of *T. asahii* at varying shaking speed (Table 2). However the maximum caffeine degradation (72 %) was attained at 120 rpm which then remained constant at 140 and 160 rpm. Below 120 rpm, there was a decrease in the degradation percentage.

The effect of inoculum size on caffeine degradation is shown in Table 2. The degradation of caffeine increased with increasing percentage of the inoculum. *T. asahii*, showed 80 % caffeine degradation in 120 h with an inoculum size of 4 % (w/v). The degradation of caffeine was reduced to 65 % when the inoculum size was increased to 5 % (w/v). Thus, an inoculum of 4 % (w/v) was found to be optimum for caffeine degradation .

The yeast was grown with caffeine in the concentration range of 1-12 gm/l. Caffeine degrading capability of *T. asahii* was exhibited up to a concentration of 12 gm/l (Fig. 5). Concentration higher than this showed relatively less degradation (not shown in the graph). The yeast could degrade 100, 92, 63 and 35 % of caffeine at concentrations of 2, 5, 7 and 12 gm/l respectively (Fig. 6).

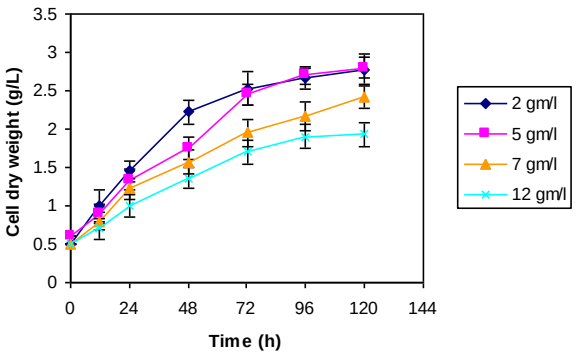

Fig. 5 Measurement of biomass of *T. asahii* grown in caffeine liquid medium containing caffeine (2-12 gm/l) and sucrose (5 gm/l) at 120 rpm, pH 6.5 and 28 °C.

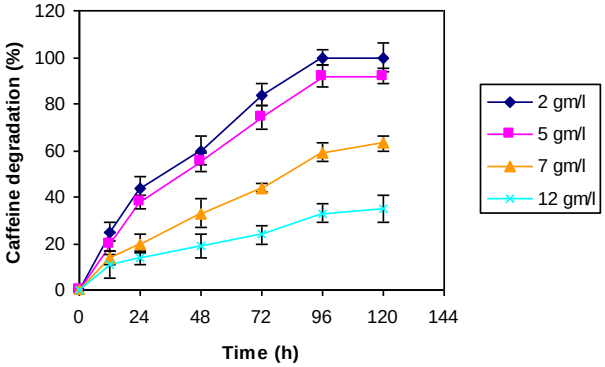

Fig. 6 Effect of caffeine concentration on degradation by *T. asahii* grown in caffeine liquid medium containing sucrose (5 gm/l) at 120 rpm, pH 6.5 and 28 °C.

Caffeine containing effluents are often discharged to the surrounding water bodies and subsequently, caffeine was detected in surface water, ground water and waste water effluents at a high concentration (~10 gm/l) (Buerge et.al., 2003; Glassmeyer et.al., 2005). The yeast isolate *T. asahii* studied here could withstand and degrade caffeine of concentration upto 12 gm/l, can thus be efficiently used for decaffeination process to remove caffeine from industrial effluent.

Yeast has many other advantages. They not only grow rapidly like bacteria, but they also have the ability to resist unfavourable environments like filamentous fungi. Furthermore, yeasts such as *Candida halophila* and *Rodotorula glutinis* have been reported as efficient agents in treating high-strength organic wastewaters, such as food industry effluents (Yang et.al., 2003).

So far our knowledge, study involving yeast as degrader of caffeine is seldom known. The degradation of caffeine under optimized conditions can be proved useful in generation of important metabolites or can be used to recover enzymes for development of biodecaffeination process. Present study may set the stage for future characterization studies of intracellular enzymes involved in caffeine degradation. Moreover, results obtained may help in the biotreatment of residues from the coffee processing industry.

Table 2 Effect of various growth parameters on caffeine degradation by *T. asahii*

Time (h)	Amount of caffeine degraded (%)±standard deviation about the mean					Cell dry weight (gm/l) ±standard deviation about the mean				
	Effect of various initial pH									
	pH 4	pH 5	pH 6	pH 6.5	pH 8	pH 4	pH 5	pH 6	pH 6.5	pH 8
0	0	0	0	0	0	0.11±0.01	0.12±0.02	0.11±0.05	0.11±0.05	0.13±0.04
24	15±5	17±4	20±5	25±6	10±8	0.30±0.02	0.30±0.01	0.36±0.01	0.47±0.04	0.26±0.03
48	17±7	25±6	30±5	54±5	14±7	0.90±0.01	1.09±0.01	1.20±0.04	1.30±0.05	0.90±0.01
72	18±7	36±5	39±7	64±6	15±7	1.35±0.01	1.45±0.01	1.50±0.03	1.56±0.02	1.33±0.02
96	22±5	41±5	50±7	67±6	17±7	1.99±0.05	2.00±0.04	2.10±0.05	2.40±0.01	1.20±0.04
120	25±8	45±8	55±5	69±8	20±8	2.30±0.02	2.41±0.04	2.50±0.02	2.65±0.01	1.45±0.04
	Effect of various temperature (°C)									
	20	25	28	35	40	20	25	28	35	40
0	0	0	0	0	0	0.13±0.04	0.12±0.02	0.12±0.04	0.12±0.04	0.12±0.02
24	20±7	25±8	27±8	20±5	10±8	0.39±0.02	0.40±0.04	0.52±0.02	0.40±0.03	0.19±0.01
48	32±6	33±6	37±7	29±5	20±7	0.99±0.04	0.99±0.05	1.10±0.04	0.80±0.01	0.50±0.05
72	45±8	45±5	48±7	40±5	25±7	1.02±0.01	1.25±0.03	1.34±0.03	1.01±0.02	0.70±0.04
96	50±7	54±7	67±6	51±5	38±7	1.50±0.05	1.99±0.01	2.34±0.01	1.40±0.04	0.84±0.05
120	59±8	60±8	73±8	55±6	40±5	1.78±0.02	2.24±0.04	2.85±0.02	1.70±0.01	0.90±0.02
	Effect of shaking speed (rpm)									
	80	100	120	140	160	80	100	120	140	160
0	0	0	0	0	0	0.12±0.03	0.13±0.04	0.13±0.03	0.13±0.02	0.12±0.03
24	20±8	22±5	25±6	25±7	20±6	0.39±0.02	0.40±0.03	0.48±0.01	0.42±0.03	0.40±0.02
48	39±5	50±6	55±6	52±5	42±7	0.97±0.05	1.20±0.04	1.30±0.05	1.25±0.01	0.98±0.05
72	50±6	60±7	65±5	65±6	60±8	1.30±0.01	1.38±0.02	1.56±0.02	1.57±0.02	1.28±0.01
96	60±7	65±7	69±5	67±6	65±7	2.10±0.01	2.20±0.05	2.60±0.01	2.50±0.05	2.23±0.05
120	64±8	67±8	72±7	70±5	70±5	2.30±0.03	2.42±0.03	2.77±0.02	2.75±0.04	2.45±0.02
	Effect of inoculum size in % (w/v) (based on wet weight)									
	1	2	3	4	5	1	2	3	4	5
0	0	0	0	0	0	0.11±0.02	0.12±0.04	0.14±0.03	0.15±0.02	0.16±0.02
24	22±5	22±8	31±7	40±5	25±8	0.32±0.01	0.41±0.02	0.62±0.03	0.90±0.01	0.78±0.03
48	30±5	34±7	45±6	58±6	48±5	0.96±0.04	0.99±0.01	1.23±0.05	1.45±0.01	1.00±0.05
72	34±7	40±7	53±5	70±6	56±7	1.00±0.04	1.30±0.01	1.50±0.02	1.80±0.02	1.20±0.01
96	44±7	59±5	66±7	75±5	61±5	1.50±0.05	1.66±0.04	1.99±0.01	2.87±0.05	2.13±0.05
120	48±6	62±6	70±6	80±5	65±6	1.71±0.02	1.93±0.02	2.50±0.04	3.39±0.04	2.20±0.02

CONCLUSION

The yeast isolate, *T. asahii* being reported by us is an efficient caffeine degrader, which may be useful in the development of an environment friendly biodecaffeination process. Degradation efficiency was found to be greatly influenced by the factors viz. pH, inoculum size and additional carbon sources. The present study helped us to understand that caffeine degradation may be enhanced by additional carbon source like sucrose whereas addition of external nitrogen sources can inhibit the degradation process. The present knowledge concerning the degradation of caffeine using yeast species as potent

degrader can be applied to the real situation where degradation is necessary in caffeine containing coffee industrial wastes.

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